



THE UNIVERSITY *of* EDINBURGH

## Edinburgh Research Explorer

### Characterisation and expression analysis of the chicken interleukin-7 receptor alpha chain

**Citation for published version:**

van Haarlem, DA, van Kooten, PJ, Rothwell, L, Kaiser, P & Vervelde, L 2009, 'Characterisation and expression analysis of the chicken interleukin-7 receptor alpha chain', *Developmental and Comparative Immunology*, vol. 33, no. 9, pp. 1018-1026. <https://doi.org/10.1016/j.dci.2009.05.001>

**Digital Object Identifier (DOI):**

[10.1016/j.dci.2009.05.001](https://doi.org/10.1016/j.dci.2009.05.001)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Publisher's PDF, also known as Version of record

**Published In:**

Developmental and Comparative Immunology

**Publisher Rights Statement:**

Copyright 2009 Elsevier Ltd

**General rights**

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

**Take down policy**

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact [openaccess@ed.ac.uk](mailto:openaccess@ed.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.





## Characterisation and expression analysis of the chicken interleukin-7 receptor alpha chain

Daphne A. van Haarlem<sup>a</sup>, Peter J.S. van Kooten<sup>a</sup>, Lisa Rothwell<sup>b</sup>, Pete Kaiser<sup>b</sup>, Lonneke Vervelde<sup>a,\*</sup>

<sup>a</sup> Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Yalelaan 1, 3584 CL Utrecht, The Netherlands

<sup>b</sup> Institute for Animal Health, Compton, Berkshire RG20 7NN, UK

### ARTICLE INFO

#### Article history:

Received 31 March 2009

Received in revised form 1 May 2009

Accepted 3 May 2009

Available online 22 May 2009

#### Keywords:

Cytokine

T cell memory

T cell activation

Immune response

Interleukin receptor

### ABSTRACT

Interleukin-7 (IL-7) is a central regulator of T cell survival and homeostasis and its expression is indicative for naïve and memory T cells. We cloned chicken IL-7R $\alpha$  (CHIL-7R $\alpha$ ) and determined its expression profile in chicken lymphocyte subpopulations. The predicted protein sequence contained 460 amino acids. The extracellular domain exhibited features typical of a type I cytokine receptor; a fibronectin type III domain and the GXWSXWS motif were conserved. ChIL-7R $\alpha$  mRNA is highly expressed in lymphoid organs and in CD4<sup>+</sup>, CD8 $\alpha$ <sup>+</sup> and CD8 $\beta$ <sup>+</sup> cells. A monoclonal antibody was generated and expression of the protein investigated. ChIL-7R $\alpha$  was expressed on CD4<sup>+</sup> and CD8 $\alpha$ <sup>+</sup>, but not CD8 $\beta$ <sup>+</sup>, T cells, in contrast to the high mRNA expression levels in all of these cells. Upon polyclonal stimulation with ConA, IL-7R $\alpha$  was rapidly down-regulated on T cells, suggesting that in the chicken expression of this receptor might also be correlated to the T cell activation status.

© 2009 Elsevier Ltd. All rights reserved.

### 1. Introduction

Mammalian interleukin-7 (IL-7) is a cytokine involved in the regulation of lymphopoiesis, acting primarily on cells of the lymphoid lineage. IL-7 was originally defined by its ability to stimulate the proliferation of pre-B cells. Cells of the T cell lineage also respond to IL-7; both foetal and adult thymocytes of all surface phenotypes proliferate in response to IL-7 [1,2].

More recently it has become evident that IL-7 is a prototypic homeostatic cytokine, produced constitutively by non-lymphoid cells [3]. The mammalian IL-7 receptor (IL-7R) is composed of two chains: the IL-7R $\alpha$ -chain (CD127) and a common  $\gamma$ -chain ( $\gamma_c$  or CD132), shared by the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. IL-7R $\alpha$  belongs to the type I cytokine receptor family. Structurally, two fibronectin-type III (FN) domains, four conserved cysteine residues and a WS motif in the extracellular domains characterise these receptors. The mature form of murine IL-7R $\alpha$  consists of 439 amino acids (aa) and is a membrane glycoprotein with a single 25 aa transmembrane domain. The 195 aa cytoplasmic tail contains partially characterised regions; one region (A) that is rich in acidic residues, and one region (T) that contains three tyrosine residues that are conserved in mouse and man (Y401, Y449, and Y456 as numbered in the murine sequence).

In addition, a small membrane-proximal domain termed “Box 1” is conserved throughout the class I cytokine receptor family [4].

Whereas the  $\gamma_c$  is expressed on most haematopoietic cells, IL-7R $\alpha$  is almost exclusively expressed on cells of the lymphoid lineage in which it has crucial functions (reviewed in [3,5]). IL-7 has emerged as a key cytokine involved in controlling the survival of peripheral resting T cells and their homeostatic turnover. The effect of IL-7 on T cells is controlled by the expression of the IL-7R, the state of differentiation of the cell, the availability of the cytokine and whether there is concomitant T cell receptor signalling (reviewed in [3,5]). Expression of IL-7R $\alpha$  is a marker of naïve and memory T cells and it may serve to distinguish memory from effector T lymphocytes at early phases of the immune response [5–7], especially when combined with the expression of CD62L. Cell subsets described as central memory T cells (CD127<sup>hi</sup> and CD62L<sup>hi</sup>) and peripheral effector memory T cells (CD127<sup>hi</sup> and CD62L<sup>lo</sup>) can be distinguished.

Moreover, surface expression of CD127 (IL-7R $\alpha$ ) combined with CD25 (IL-2R $\alpha$ ) can differentiate between human regulatory and conventional CD4<sup>+</sup> T cells in adult peripheral blood [8]. CD127<sup>lo</sup> T regulatory cells and CD127<sup>hi</sup> conventional T cells can be distinguished within the CD25<sup>+</sup> CD45RO+RA<sup>–</sup> effector/memory and CD25<sup>+</sup> CD45RA+RO<sup>–</sup> naïve compartments. CD25<sup>+</sup>CD127<sup>lo</sup> cells have suppressive activity *in vitro* whereas CD25<sup>+</sup> CD127<sup>hi</sup> cells do not [8].

For the class I cytokine receptor family in the chicken, all of the common signalling chains have either been cloned and characterised [9,10] or are present in the chicken genome sequence

Abbreviations: IL, interleukin; chIL-7R $\alpha$ , chicken interleukin-7 receptor alpha chain.

\* Corresponding author. Tel.: +31 30 2531872; fax: +31 30 2533555.

E-mail address: [L.Vervelde@uu.nl](mailto:L.Vervelde@uu.nl) (L. Vervelde).

(reviewed in [11]). IL-7 is encoded in the chicken genome and is expressed [12]. In the chicken, naïve, effector and memory T cells cannot be distinguished yet and the presence of regulatory T cells has not been demonstrated, but tools and assays to dissect T cell subsets are becoming available [13]. Here, we describe the cloning and expression of the *chIL-7R $\alpha$*  and the development of a monoclonal antibody specific for *chIL-7R $\alpha$* , which will facilitate future research in chicken T cell development and discrimination between functional T cell subsets.

## 2. Materials and methods

### 2.1. Animals and tissues

To study mRNA expression of IL-7R $\alpha$ , liver, spleen, thymus, bursa of Fabricius and bone marrow were collected from broiler chickens (Ross) at embryonic days 7 and 20 (ED7 and ED20;  $n = 4$ ). Peripheral blood and spleens from 4-day-old to 6-week-old broiler chickens were used to sort cell subpopulations. Peripheral blood leukocytes (PBL) were isolated by density gradient centrifugation for 20 min at  $850 \times g$  using FICOLL-Hypaque 1.078. Subpopulations of leukocytes were obtained after staining of cells with mouse anti-chicken CD4-PE (CT-4), CD8 $\alpha$ -FITC (CT-8), CD8 $\beta$ -PE (EP42), and Bu-1-FITC (ChB6 clone AV20; Southern Biotechnology (SB), USA). Cells were sorted using a FACS Vantage SE instrument (Becton Dickinson). For every cell subpopulation at least  $3 \times 10^4$  PBL and  $1 \times 10^6$  splenocytes were analysed. For total RNA extractions, organs and cells were immediately immersed in RNAlater (Ambion) and stored at  $-20^\circ\text{C}$  until RNA was extracted.

### 2.2. Isolation of chicken IL-7R $\alpha$ cDNA

Total RNA was extracted in the presence of buffer containing  $\beta$ -mercaptoethanol and guanidine using an RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA was eluted in 30  $\mu\text{l}$  RNase-free water and stored at  $-20^\circ\text{C}$  until required. The extracted RNA was reverse-transcribed using an iScript cDNA Synthesis Kit (Bio-Rad), with an oligo dT primer. *CHIL-7R $\alpha$*  was amplified using Kod Hot Start DNA Polymerase (Novagene) according to the manufacturer's protocol. Briefly, cDNA (1  $\mu\text{l}$ ) was mixed with 200  $\mu\text{M}$  dNTPs,  $1 \times$  PCR buffer for Kod Hot Start DNA Polymerase, forward and reverse primers (0.3  $\mu\text{M}$ ) and 1 unit Kod Hot Start DNA Polymerase in 50  $\mu\text{l}$  final volume. PCR conditions were as follows, 1 cycle of  $94^\circ\text{C}$  for 5 min followed by 30 cycles of  $94^\circ\text{C}$  for 15 s,  $61^\circ\text{C}$  for 30 s and  $72^\circ\text{C}$  for 20 s followed by 1 cycle at  $72^\circ\text{C}$  for 5 min, using an iCycler (Bio-Rad). All primers used for the PCR were designed to the EST sequence of *chIL-7R $\alpha$*  in the chicken genome. Two primers, *chIL-7R* forward (5'-AGTATAAAGTCAGTGAGTGAGGGTTTC-3') and *chIL-7R* reverse (5'-AATAGAGAGCTTCAGTATCCAGCTTG-3') were designed outside the predicted *chIL-7R $\alpha$*  coding sequence. To increase specificity of the PCR the first PCR product was used for a nested PCR. To obtain the full-length cDNA sequences for *chIL-7R $\alpha$* , two primers, *chIL-7R*-nested forward (5'-GGGGAATTCAGCATGCTCAGAATGACACG-3') and *chIL-7R*-nested reverse (5'-GGGCTAGAAACGCTACT-

GATTTTGTAAAGC-3') were used in PCR. The forward primer contains an EcoRI restriction site at the 5'-end and the reverse primer contains a XbaI restriction site at the 3'-end. These restriction sites were used to ligate the PCR product into pCI-neo (Promega) and pcDNA3.1 myc-his (Invitrogen). Library-competent *Escherichia coli* DH5 $\alpha$  cells (Invitrogen) were transfected with the plasmids using FuGene-6 Transfection Reagent (Roche) according to the manufacturer's protocol. Plasmid DNA was extracted with a Quantum Prep Plasmid Maxiprep Kit (Bio-Rad). The insert DNA was sequenced using the primers T7 promoter (5'-TAATACGACTCACTATAGGG-3') and BGH-Reverse (5'-TAGAAGGCACAGTTCGAGG-3') for pcDNA3.1 myc-his and T7EEV (5'-AAGGCTAGAGTACTTAA-TACGA-3') and T3 (5'-ATTAACCTCACTAAAGGGA-3') for pCI-neo.

Alignment of various IL-7R $\alpha$  aa sequences was performed using the ClustalW Chicken FN\_III domain prediction was performed using Pfam (<http://www.sanger.ac.uk/software/pfam/>). The growth factor and cytokine receptors family signature 2 (GFCRF52) motif was predicted by Prosite motif search (<http://www.expasy.ch/prosite>). Similarity searches were performed using Blast Alignment (NCBI: <http://www.ncbi.nlm.nih.gov/>).

### 2.3. Chicken IL-7R $\alpha$ expression analysis by real-time quantitative RT-PCR

IL-7R $\alpha$  mRNA levels in different organs and cell subpopulations were quantified using real-time quantitative RT-PCR (qRT-PCR). For both cytokine receptor- (Accession No. EF116487) and 28S rRNA-specific amplification, primers and probes were designed using the Primer Express software program (PE Applied Biosystems). Details of probes and primers are given in Table 1.

QRT-PCR was performed using the TaqMan Universal PCR Master Mix (PE Applied Biosystems). Amplification and detection of specific products were performed using the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems) with the following amplification profile: 1 cycle of  $50^\circ\text{C}$  for 2 min,  $95^\circ\text{C}$  for 10 min and 40 cycles of  $95^\circ\text{C}$  for 20 s,  $59^\circ\text{C}$  for 1 min. Primers were used at 600 nM and probes at 100 nM. Quantification was based on the increased fluorescence detected by the ABI-PRISM 7700 Sequence Detection System due to hydrolysis of the target-specific probes by the 5' nuclease activity of the AmpliTaq Gold DNA Polymerase during PCR amplification. A passive reference dye ROX (present in the TaqMan Universal PCR Master Mix), which is not involved in amplification, was used to correct for fluorescent fluctuations resulting from changes in the reaction conditions, for normalisation of the reporter signal.

Results are expressed in terms of the threshold cycle value (Ct), the cycle at which the change in reporter dye passes the significance threshold ( $\Delta\text{Rn}$ ). Corrections for variations in RNA preparation and sampling were performed according to Eldayes et al. [14]. Results were then expressed as 40-Ct.

### 2.4. Expression *chIL-7R $\alpha$* on leukocytes

Two Balb/c mice were immunised three times with 50  $\mu\text{g}$  plasmid DNA, pCI-neo-*chIL-7R $\alpha$* , at three-week intervals. After

**Table 1**  
Real-time qRT-PCR probes and primers.

RNA target	Probe/primer sequence (5'-3')	Accession No.
IL-7R $\alpha$	Probe F R (FAM)-TCGTGTGGCCAAACCTTCCTGATCAT-(TAMRA) ATTCTGGGAAAGCAGGATCAAG CTTACACAGTCGCTCCAGAGTTATTT	EF116487
28S	Probe F R (VIC)-AGGACCGCTACGGACCTCCACCA-(TAMRA) GGCGAAGCCAGAGGAACT GACGACCGATTGACGCTC	X59733

ATGCTCAGAATGACACGGATGAGTACAGTATTGAGCATTTTCATCTTGTTCCTTCATACC  
 M L R M T R M S T V L S I F I L F L H T  
 ACTTTTGGGGAAAGTGGTTGCACCTCAGCAGATGGTGATGGGACCTTTGGAGATGACGAA  
 T F G E S G C T S A D G D G T F G D D E  
 CCTGATAATTTTGACATTGACTGCTTCAGCCAGCTGGAATTTAAGGATTCCTACAGCAGC  
 P D N F D I D C F S Q L E F K D S Y S S  
 CTGACCTGCAATTTTACAGAGCTGCCTCCTCACAACACTAACTATACCCTGGCTGTGTGT  
 L T C N F T E L P P H N T N Y T L A V C  
 ACCAAGGAAGACAGCAGTTACCTGTGTTTCAATATGGAGAAACAGGAAGATGTTTATTTT  
 T K E D S S Y L C F N M E K Q E D V Y F  
 TTACAGTTCACCGATATACTCTCAAATAAAGACATTTGTGTGGAATATGAGATAAAGAGA  
 L Q F T D I L S N K D I C V E Y E I K R  
 AGGGCCTGCAGGAGTCTGATTGTAACCTGACATTGTCAAGCCTGAGGTACCATTTGACATA  
 R A C R S L I V T D I V K P E V P F D I  
 AACATAACATACCAAAGGAGGCAAATGAATATCTCATTTCATTATAGTACACCACACTCA  
 N I T Y Q K E A N E Y L I H Y S T P H S  
 CGGAAGAAATACTTAAAGGACAAATTAATACATCAGATAGCCTACCGGCAGGAAGAAAGC  
 R K K Y L K D K L I H Q I A Y R Q E E S  
 ACTTGAAGACCATAAAATCACCATACCTTCAGGTAAAGTTACTGGGAAAAAACCTCGAA  
 T W K T I K S P Y L Q V K L L G K N L E  
 GCAGATGCGTTGTATGAGGTGAAAGTGCCTTCCAGCCCAACGGAGATTACTTTAAGGGC  
 A D A L Y E V K V R S Q P N G D Y F K G  
 ATATGGAGCGAGTGGAGCACTTCGAAGAGCTTCAGGACCACAGGAGAGCACTCCATGGAG  
 I W S E W S T S K S F R T T G E H S M E  
 AGCTATAGCAGCATGTTTGTGATTATACTCTCCATTCCAGGATTCATTCTGTCTGAGTTGTC  
 S Y S S M F V I I L S I P G F I L S V V  
 ATGATTGTCTCTGATCCTAACATTCTGGGAAAGCAGGATCAAGCCTGTCTGTGGCCAAAC  
 M I V L I L T F W E S R I K P V V W P N  
 CTTCTGATCATATAAAATAACTCTGGAGCGACTGTGTAAGAGGCCAAAAAATAATTTTGAT  
 L P D H K I T L E R L C K R P K N N F D  
 ATAAGCTTTTAACCCAGAGAGTTTGGTTATGTTTTTATCCATGAAGTGGATGGCATTCAA  
 I S F N P E S F G Y V F I H E V D G I Q  
 GCAAAAGCCGAACAGGAAAAATTTCTGCAGCCACCACCTACTCCAGAGACAGATATTCCA  
 A K A E Q E N F L Q P P P T P E T D I P  
 CCAAAGTTTAGGAGTGGATCAGACTTGAAGCGAAGTCCTGCAAGGATTGACAAAAACAGC  
 P K F R S G S D L K R S P A R I D K N S  
 CTGAACCTGTCTGTGAGTTATGGAGGAATCTGGCCTGCAGAGGCTCTTCATGGACTCTTT  
 L N L S V S Y G G I W P A E A L H G L F  
 GGTTCAGCCAGTCTACAGCTGCTGATGTCTGCAGCTCTGGCACATACGAGGTGTGCCAC  
 G C S Q S T A A D V C S S G T Y E V C H  
 AGCAGCAGAGTGCCTCTGTGTGACAATGGCTTTACCTTCCTTCTGCTCCTCCCTTGGAT  
 S S R V P L C D N G F H L P S A P P L D  
 CCTCCAGGACAACCTGGACCACAGCCCCAGAATGGCAACGTGGTGCCACCTAACAGTGAA  
 P P G Q P G P Q P Q N G N V V P P N S E  
 TTGAAGTACCGAGTGATGAAGAAGCCTATGTCCACCATGTCTGAGCTTTTACAAAAATCAG  
 L K S P S D E E A Y V T M S S F Y K N Q  
 TAG  
 \*

Fig. 1. Nucleotide sequence and predicted amino acid sequences of chicken IL-7R $\alpha$ . Different font colours indicate the different exons.



three weeks, mice were boosted with  $2 \times 10^7$  chicken thymocytes. Hybridomas were generated by fusion of splenic lymphocytes, two weeks after the last boost, with mouse myeloma Sp2/0–Ag14 cells, according to the method of Segers et al. [15]. After fusion, the cells were distributed in flat-bottomed 96-well plates. Supernatants from wells containing growing colonies were tested by indirect immunofluorescence on chicken PBL and measured by flow cytometry. The second round of selection also included reactivity on spleen cryosections using immunohistochemistry [16] and on

COS cells expressing recombinant chIL-7R $\alpha$ . Positive wells were cloned by single cell limiting dilution on the FACSvantage cell sorter (BD Biosciences, San Jose, CA) using CellQuest software (BD Biosciences). Immunoglobulin subclass was determined using a Beadlyte Mouse Immunoglobulin Isotyping Kit (Upstate) using the Luminex 100 System.

IL-7R $\alpha$  expression at the protein level was measured on PBL and splenocytes from Heisdorf & Nelson Silver Nick layer birds of one, three and six weeks of age. Blood was diluted in pre-warmed

chicken	: MLRMTRVSTVLSIFILFHTTFGESECTSADGDGTFGDEPDNFDIDCFSQLB-FKDSYS	: 59
human	: ---MTILGTTFGMVFSLLQVVSAGESYAQN---GDLEDAELDDYSFSCYSQLE-VNGSQH	: 53
mouse	: ---MMAIGRAFAIVFCLIQAVSAGESNAQD---GDLEDAADHDSFWCHSQLB-VDGSQH	: 53
dog	: ---MTILGTAFGMVFYLLQAVSAGESYAQN---GDFEDAELDDYSFSCYSQLE-VDGTQH	: 53
cow	: -MRMTLSIALGTFLFYLLQAVSAGESYAEN---GDFEDAELDDYSFSCYSQLE-VDGLQH	: 55
zebrafish	: -----MQLILY-CFPNDEPIEEVIF	: 19
chicken	: SLTCTELPPHNTNYTLAVCTKEDSSYLQFNMEKQEDVYFLOFTDILSN--KDICTVEYE	: 117
human	: SLTCAEDPDVNITNLEFEICG-ALVEVKCLNFRKIQEIFYETKKFELLIGKSNICVKVG	: 112
mouse	: LLTCAEDSDINTANLEFQICG-ALLRVKCLTLNKLQDIYFKTSEFELLIGSSNICVKLG	: 112
dog	: LLTCTEDDPDINSTNLEFEICG-ALLNVKCLTFNKKQEMFYFKTKKELLIGDSEIYVKLE	: 112
cow	: LLTCEDDSDINSTNLEFEICG-ALLVDCLNFNKLQERYFKTKKELLIGDSKICVKLE	: 114
zebrafish	: ATLCKMKEKSK-----CTNATLGPKYFSFENLENIYKVELQVHLRDC-----	: 61
chicken	: IKRRACRSLLIVTDIVKPEVPFDINTIYQKEANEYLIEYSTPHSRKKYLKDKLIHQIAYRQ	: 177
human	: EKSLTCKKIDLTIVKPEAPFDLSVVYREGANDFVVTENTSHLQKKYVK-VLMHDVAYRQ	: 171
mouse	: QKNLTCKNMALNTIVKAEAPSDIKVYRKEANDFLVTFNAPHLKKYK-KVKHDVAYRP	: 171
dog	: GKNITSKKNLTVIRIVKPEAPDVRVYRKEANDFLVTENTSHLQKKYVK-ELVHEVAYRQ	: 171
cow	: KKNMTCKRVNIVKIVKPEAPDVRVYRKEANDFVVTENTSHLQKKYVK-VLTHEVAYRQ	: 173
zebrafish	: ---IIEKEIDLTKMVKIPABELKSATLKETDEIFLWFEHRH---DYVR---QCQFQVE	: 111
<u>GFCRFS2</u>		
chicken	: E--ESTVKTIKSPYLQVLLGHNLEADALYEVKVRSCENGDYFKGIWSEWSTKSFRTTG	: 235
human	: EKDENKTHVNLSSSTKLTLLQRLQPAAMYELKVRSLP-DHYKGFSEWSEWSPSYFFRTPE	: 230
mouse	: ARGESNATHVSLFHTRTIPQKRLRPKAMYELKVRSLPHNDYFKGFSEWSEWSPSTFFRTPE	: 231
dog	: EKNENDTCVNLSSSTKLTLLQRLQPDAMYELKVRSLPNANYFQGFSEWSEWSPSFHFRTPPE	: 231
cow	: EKNENNMRVNLSTKLTLLQRLQPNAMYELKVRSLPYANYFEGFSEWSEWSPSFHFRTPPE	: 233
zebrafish	: IRGEHEPISLLVDYKNISMSRDLGGDGVYSTRVRAKE-INYFAGDSEWSSDANFTIKT	: 170
<u>Box 1</u>		
chicken	: EHSMESYSSMFVILSLPGFILSVVMIVLILTFWESRIKPVVWPNLPDHKKTTLERLCKRP	: 295
human	: INNSSGEMDPILLTISLSFFSVALLVILACVLWKKRIKPIVWPSLPDHKKTLEHLCKKP	: 290
mouse	: PKNQG-GWDPVLPSTVILSLFSVLLVILAHVLWKKRIKPVVWPSLPDHKKTLEQLCKKP	: 290
dog	: ITGR---MDPVLLTISLSFFSVTLMLVILAFALWKKRIKPMVWPSLPDHKKTLEQLCKKP	: 288
cow	: TNGTG-ETDPVLLTISLSFFSVALLMVTLACVLWKKRIKPIIWPSPDPHKKKTLEQLCKKP	: 292
zebrafish	: KNHAP---IQTPQVVTGLTIIIVLIIISLIALRWRTQIKKYITPNIHPKATLAQMOR-A	: 226
chicken	: KKNFDLSFNPESEFGYVFIHEVDGIQAKAEQENFLQPPPTPETDIPPKFRSGSDLKRSFAR	: 355
human	: RKNLNVSNFPESEFLDCQIHRVDIQRDEVEGFLQDTFFQLESEKQRLGGDVQ-SPNC	: 349
mouse	: KTSNLNVSNFPESEFLDCQIHEVKGVEARDEVESEFLPNDLPAPEELETQGHRAVH-SANR	: 349
dog	: KKNLNVSNFPESEFLDCQIHKVDGIQARDEAESEFLQDTSP-QLDESEKQRLGGGLQ-GPTW	: 346
cow	: KKNLNVSNFPESEFLDCQIHKVDGIQARDEAESEFLQDPLPPQPEESDKORFRAGMQ-GPSW	: 351
zebrafish	: KEGLPFTFSPETESDAFIHRVDYVDEK-----PSAPQLQDELRYSS-----S	: 267
chicken	: IDKNSLNLVSYSYGGIWEAEAHGLFGCSQSTAADVCSSTGYEVCHSSRVPLCDNGFHLPS	: 415
human	: PSEDVITPESFGRDSSLTCLAGNVSAADAPILSSSRSLDCRESGKNGPHVYQDLLLSLG	: 409
mouse	: SPETSVPPTETVRRESLRLCLARNLSTCNAPPILSSRSPDYRDGDRNRPPVYQDLLPNSG	: 409
dog	: PPEHAVITPKTFRGDPEFGCLAGNVSMFEALAPSFRLADCREGGKNGPHVYQGLLLGPG	: 406
cow	: PSEQAGITPKIFRGESFRLCLAGNASVCDAPGLSSRSPNGREGGKSRPLVYQDLLLPG	: 411
zebrafish	: SSASVSEMDMNADERLREQSHLKLRLLEDSELEKEMENSVSQG-----	: 311
chicken	: APPLDPPGQPGPQPNQNVVP-----PNSLKSQPSDEEAYVTMSSFYQNK-	: 460
human	: TTNS--TLPPPSFLQSCILTLNPVAQGPILTLGSSQEEAYVTMSSFYQNK-	: 459
mouse	: NTNVPVPVPQPLPFQSCILIP--VSQRQPISTSSVLNQEAYVTMSSFYQNK-	: 459
dog	: TTSG--TVPTFPFQSCILTPNPAAGQPVLTSLGSSQEEAYVTMSSFYQNK-	: 456
cow	: TTNS--SLPPFPFQSCILTLNPVAQGPILTLGSSQEEAYVTMSSFYQNK-	: 461
zebrafish	: -----LKSQPLECKEAYVTMSSFLFKTK-	: 334

**Fig. 2.** Comparison of amino acid sequences between chicken IL-7R $\alpha$  and human, mouse, dog, cow and zebrafish IL-7R $\alpha$  (Accession numbers EF116487, NP\_002176, EDL03312, XP\_855408, XP\_599818 and NP\_001106979 respectively). The predicted Fn\_III domain is underlined. The conserved GFCRFS2 (growth factors and cytokine receptors family signature 2) domain (GXWSXWS motif) in the Fn\_III domain is overlined and annotated. The predicted trans-membrane domain is double-underlined. In the cytoplasmic tail, a small membrane-proximal domain, termed "Box 1" (Val<sup>277</sup> to His<sup>284</sup>) is also overlined and annotated. A conserved 11 aa motif at the COOH-terminal of the protein, containing two tyrosine residues (Tyr<sup>450</sup> and Tyr<sup>457</sup>), is double-overlined.

Hanks Balanced Salt Solution and lymphocytes purified after slow-spin centrifugation ( $62 \times g$ , 20 min, RT). PBL and splenocytes were triple-stained with mouse anti-chicken IL-7R $\alpha$  and goat anti-mouse (IgM)-APC (SB), fluorescein (FITC)-labeled or phycoerythrin (PE)-labeled mouse anti-chicken CD8 $\alpha$ , CD8 $\beta$ , CD4, ChB6 (Bu-1-FITC), or KUL01-FITC (monocyte/macrophages; SB) and with T cell receptor-specific antibodies TCR- $\gamma\delta$ -FITC (TCR1), TCR- $\alpha\beta$ 1-FITC (TCR2), or TCR- $\alpha\beta$ 2-FITC (TCR3; SB). Cells were acquired on a FACSCalibur flow cytometer (BD Biosciences) and analysed using CellQuest software.

Splenocytes were stimulated for 24 h at 41 °C with 5 or 10  $\mu$ g/ml ConA or medium (RPMI-1640, 5% FCS, penicillin, streptomycin) and triple-stained for chIL-7R $\alpha$  and T cell markers as described above.

Immunocytochemical stainings were performed on cryosections of spleen and thymus. Sections were acetone fixed and incubated with monoclonal antibodies specific for chIL-7R $\alpha$  (biotin labeled) and ChB6 (Bu-1), CD4, CD8 $\alpha$ , CD8 $\beta$ , TCR- $\alpha\beta$ 1, TCR- $\alpha\beta$ 2, TCR- $\gamma\delta$  or KUL01 (monocytes and macrophages). Slides were washed and incubated with avidin–enzyme complex (Vectastain<sup>®</sup> Elite ABC kit) and goat anti-mouse IgG-AP. Alkaline phosphatase activity was developed with Fast Blue BB salt and then the peroxidase activity was developed with AEC [17].

### 3. Results

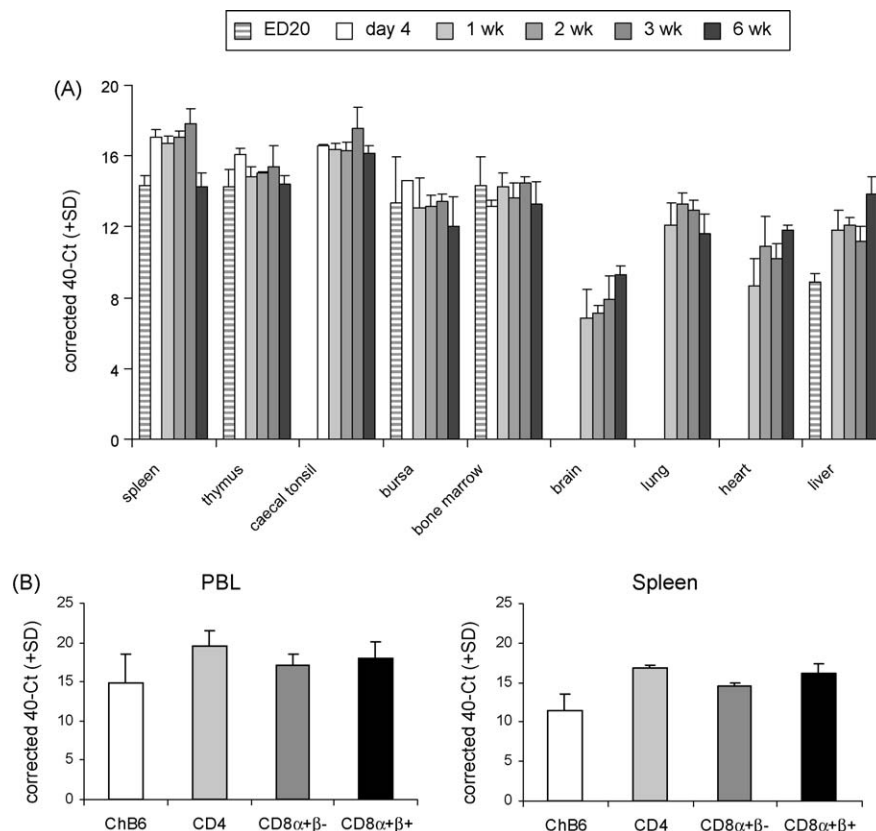
#### 3.1. Cloning and characterisation of full length chIL-7R $\alpha$

Using a PCR-based approach with primers designed to the predicted sequence in the chicken genome, we cloned the *CHIL-7R $\alpha$*  chain cDNA (Fig. 1). The predicted protein sequence of chIL-7R $\alpha$  contained 460 aa residues (Fig. 1), and included a predicted

cleavable signal peptide (Met<sup>1</sup> to Gly<sup>23</sup>), and a calculated molecular mass of 52.2 kDa. The predicted aa sequence of chIL-7R $\alpha$  was compared with those of human, mouse, rat, dog, tetraodon and fugu (Fig. 2). Three cysteine residues and the GXWSXWS motif (aa 220–226) located in the fibronectin type III domain (Pro<sup>134</sup> to Ser<sup>223</sup>) were conserved in all species. Another four cysteine residues were conserved with mammalian species, except the dog, where only two were conserved. ChIL-7R $\alpha$  showed 34.4% identity to human IL-7R $\alpha$  at the aa level. The predicted transmembrane domain was 24 aa in length, from Val<sup>247</sup> to Glu<sup>270</sup>. The cytoplasmic tail contains regions that are partially characterised in human and mouse. A small membrane-proximal domain, termed “Box 1” (Val<sup>277</sup> to His<sup>284</sup>), was highly conserved. An 11 aa motif at the COOH-terminal of the protein, containing two tyrosine residues (Tyr<sup>450</sup> and Tyr<sup>457</sup>), was completely conserved between the chicken and mammalian species. Four potential N-linked glycosylation sites (NXS/T) were located at aa 64–66, 74–76, 141–143, and 362–364 in chIL-7R $\alpha$ , compared to nine in human IL-7R $\alpha$ .

#### 3.2. Expression of chIL-7R $\alpha$ mRNA

Quantification of RNA levels was performed using real-time qRT-PCR and normalised against 28S rRNA expression levels. Expression of chIL-7R $\alpha$  mRNA was analysed in organs of ED20 embryos and in chickens post-hatch (Fig. 3A). In both embryos and chickens post-hatch, expression was seen in all organs, with highest levels of chIL-7R $\alpha$  mRNA detected in lymphoid organs which contain a high number of T cells. Expression of chIL-7R $\alpha$  mRNA was detected as early as ED7 in pooled lymphoid organs and in the yolk sac, though expression levels were lower than at ED20 (data not shown). Lymphocyte subpopulations from blood and spleen of 6-week-old birds were sorted and analysed (Fig. 3B). In



**Fig. 3.** A. Quantification of chIL-7R $\alpha$  mRNA extracted from lymphoid and non-lymphoid organs of birds of different ages. Results are expressed as the mean corrected 40-Ct values +SD of 4 birds per time-point. B. PBL and splenocyte subpopulations were sorted using a FACSVantage and chIL-7R $\alpha$  mRNA expression levels in the different subpopulations were quantified. Results are expressed as the mean corrected 40-Ct values +SD of at least 3 chickens.

the spleen, expression of *chIL-7R $\alpha$*  did not vary much between chickens and significantly higher expression of *chIL-7R $\alpha$*  mRNA was found in T cells compared to B cells ( $p < 0.05$ ).

### 3.3. Expression of *chIL-7R $\alpha$* on leukocytes

After several rounds of selection, one hybridoma (8F10E11) was selected that reacted with PBL using flow cytometry, on cryosections of spleen using immunohistochemistry, and on transfected COS cells. This clone was found to be secreting an IgM antibody with a kappa light chain and was used for further characterisation and expression of *chIL-7R $\alpha$*  on leukocytes.

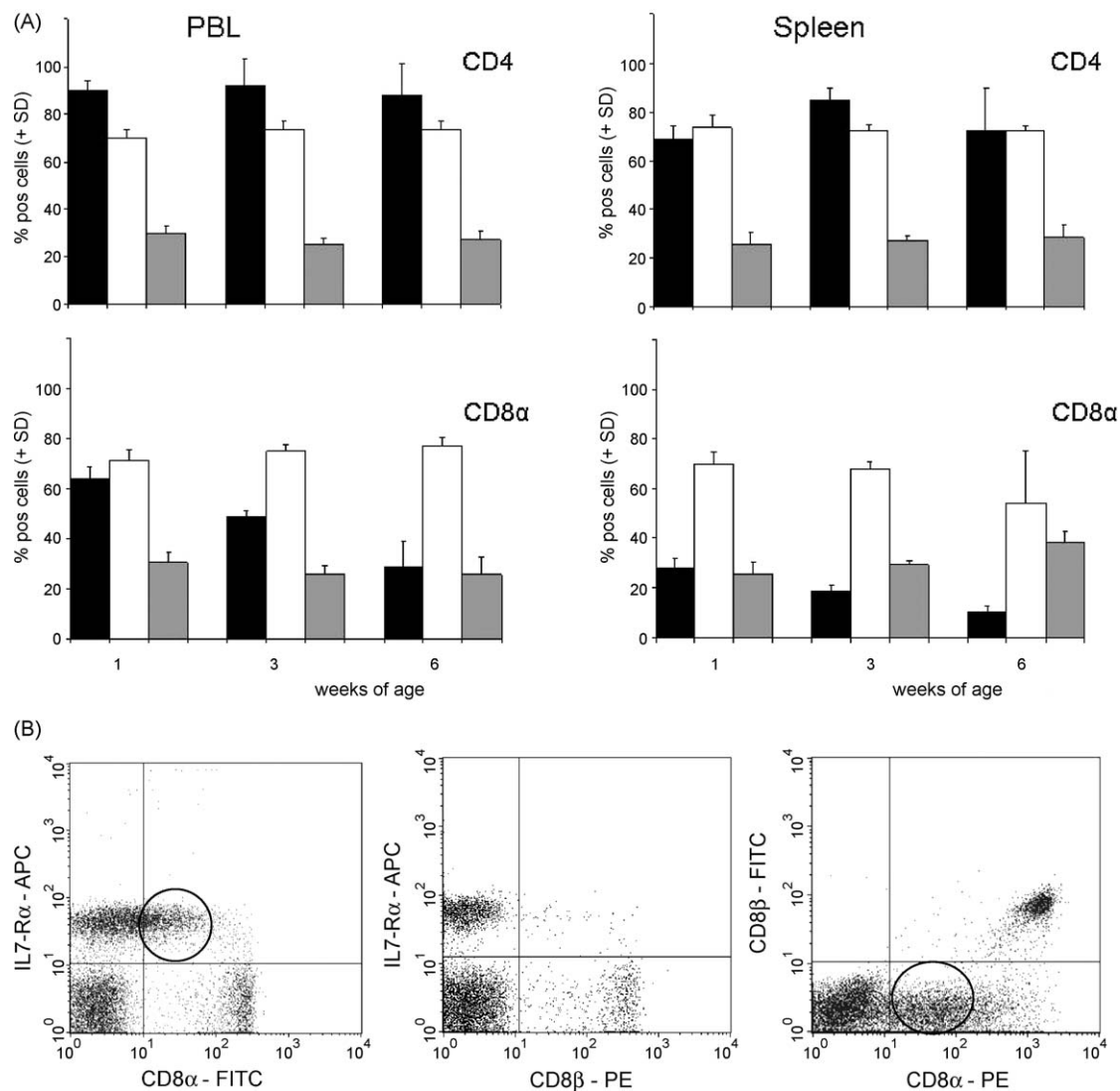
Age-related expression of *chIL-7R $\alpha$*  was determined on PBMC and splenocytes of birds of one, three and six weeks of age. Although *chB6+* B cells expressed *chIL-7R $\alpha$*  at the mRNA level, the percentage of *chB6+* B cells that expressed the protein at the membrane surface was very low ( $<1\%$ ; data not shown). On monocytes and macrophages (*KUL01+* cells), *chIL-7R $\alpha$*  expression was also rare ( $<1\%$ ; data not shown). No changes with age in blood and spleen were found for either cell population.

T cells were triple-stained for *chIL-7R $\alpha$* ; CD4, CD8 $\alpha$  or CD8 $\beta$ ; and T cell receptors (Fig. 4A). In peripheral blood, 90% of the CD4+

cells expressed *chIL-7R $\alpha$*  of which 70–75% were TCR- $\alpha\beta1+$  (TCR2) and 25–30% TCR- $\alpha\beta2+$  (TCR3). In the spleen, 70–80% of the CD4+ cells expressed *chIL-7R $\alpha$* , with a similar distribution of the TCRs as on CD4+ cells in blood. *chIL-7R $\alpha$*  was not preferentially expressed on TCR- $\alpha\beta1+$  or on TCR- $\alpha\beta2+$  cells. The percentage of CD4+ cells that expressed *chIL-7R $\alpha$*  in both blood and spleen was not significantly affected by age.

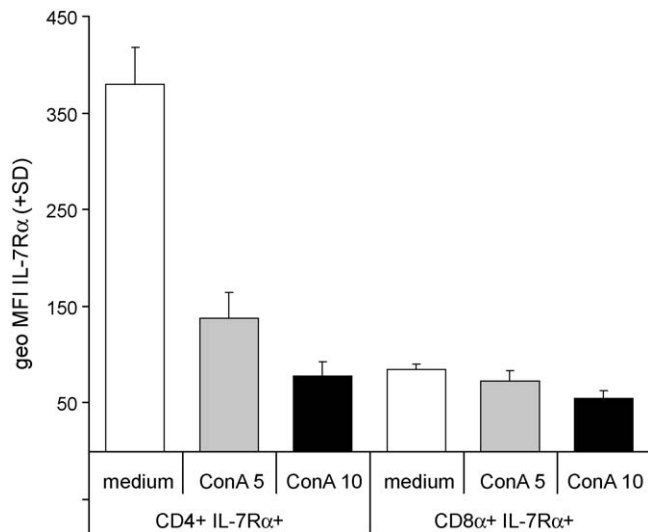
In peripheral blood, the percentage of CD8 $\alpha$ + cells expressing *chIL-7R $\alpha$*  decreased with age from 60% to 30%. Of the CD8 $\alpha$ + *chIL-7R $\alpha$* + cells in blood, 75% of cells co-expressed TCR- $\alpha\beta1$  and 25% TCR- $\alpha\beta2$ , with no significant changes with age. Fewer than 1% of the cells were TCR- $\gamma\delta+$  (data not shown). In the spleen the percentage of CD8 $\alpha$ + cells that expressed *chIL-7R $\alpha$*  was much lower than in blood, but a similar decrease with age was found, ranging from 30% to 10%. With age the percentage of CD8 $\alpha$ + *chIL-7R $\alpha$* + cells that co-expressed TCR- $\alpha\beta1$  decreased whereas the percentage of cells that co-expressed TCR- $\alpha\beta2$  increased. The percentage of CD8 $\alpha$ + *chIL-7R $\alpha$* + TCR- $\gamma\delta$ + cells was higher in the spleen than in the blood, increasing from 1% to 10% with age (data not shown).

CD8 $\alpha$  expression could be subdivided into high and intermediate expression (Fig. 4B). The expression of *chIL-7R $\alpha$*  in blood and spleen was mostly confined to the CD8 $\alpha^{\text{int}}$  cells. These CD8 $\alpha^{\text{int}}$



**Fig. 4.** A. Expression of *chIL-7R $\alpha$*  on PBL and splenocytes of 1-, 3- or 6-week-old birds, as measured by FACS analysis. Black bars show the percentage of T cells (CD4 or CD8 $\alpha$ ) that express *chIL-7R $\alpha$* . Grey bars show the percentages of CD4+ *chIL-7R $\alpha$* + or CD8 $\alpha$ + *chIL-7R $\alpha$* + cells that express TCR- $\alpha\beta1$  (TCR2; white bars) or TCR- $\alpha\beta2$  (TCR3; grey bars). Bars represent percentage positive cells within the live cell gate  $\pm$ SD ( $n = 4$ ). B. Expression of *chIL-7R $\alpha$*  on CD8 $\alpha$ + PBL. Cells of a 3-week-old bird were triple-stained for *chIL-7R $\alpha$* , CD8 $\alpha$ , and CD8 $\beta$  and FACS analysis indicated that CD8 $\alpha^{\text{int}}$  cells (circled) are CD8 $\beta$ - and *chIL-7R $\alpha$* +, whereas CD8 $\alpha^{\text{hi}}$  cells co-express CD8 $\beta$  but rarely *chIL-7R $\alpha$* .





**Fig. 5.** Splenocytes of 3-week-old birds ( $n = 4$ ) were stimulated with 5 or 10  $\mu\text{g/ml}$  ConA for 24 h. The cells were double-stained for expression of chIL-7R $\alpha$  and the T cell markers CD4 or CD8 $\alpha$ . ChIL-7R $\alpha$  expression levels are given as geometric mean fluorescent intensity (geometric MFI)  $\pm$  SD.

cells rarely expressed CD8 $\beta$ , which was in sharp contrast to the expression of chIL-7R $\alpha$  at the mRNA level. Changing the order in which the monoclonal antibodies were used to stain the cells did not affect the result, suggesting that the lack of staining of CD8 $\beta$ + cells was not due to steric hindrance of the monoclonal antibodies that were used.

Splenocytes were stimulated with a polyclonal T cell stimulus, ConA, in order to study changes in expression of chIL-7R $\alpha$  upon activation. Stimulation for 24 h resulted in a tremendous down-regulation of chIL7R $\alpha$  on CD4+ and only a slight down-regulation on CD8 $\alpha$ + T cells (Fig. 5). The number of CD4+ and CD8 $\alpha$ + cells increased after stimulation and the geometric MFI of CD4+ and CD8 $\alpha$ + cells slightly decreased (data not shown).

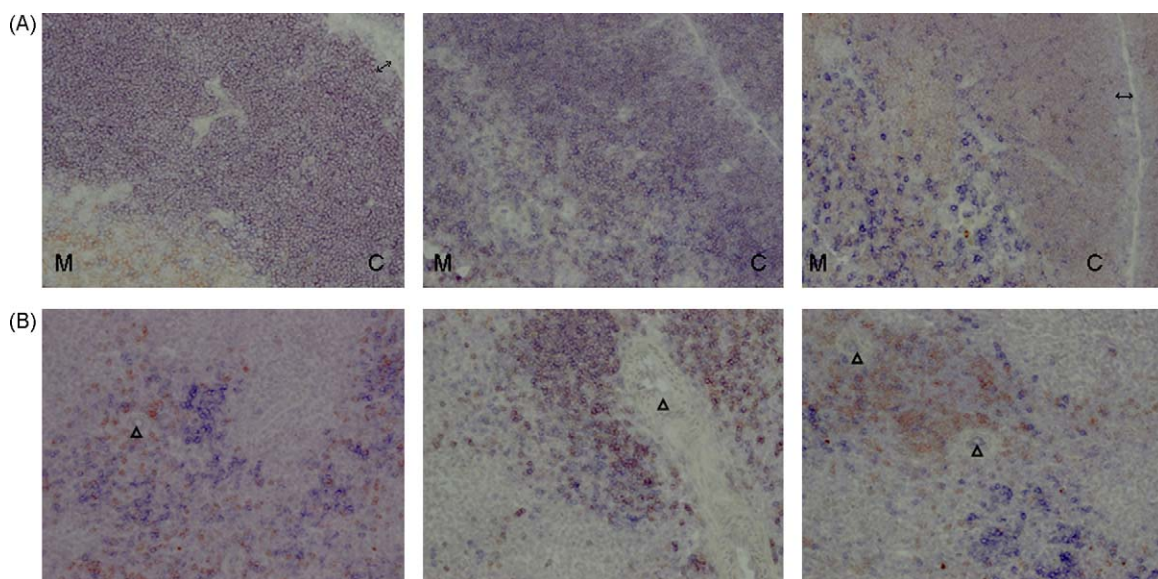
The expression of chIL-7R $\alpha$  in thymus and spleen was studied using immunocytochemistry. Cortical thymocytes mainly represent double-positive cells expressing both CD4 and CD8. ChIL-7R $\alpha$

was expressed on most cortical thymocytes except for the outer 2–5 cell layers in the subcapsular sinus, where the differentiation of the precursor cells starts. These thymocytes do not express CD4 or CD8. During migration from the cortex to the medulla, the thymocytes mature into single-positive T cells [18]. In the medulla most CD4+ and CD8+ cells co-expressed chIL-7R $\alpha$ . Differences in staining were found when cells were stained for TCR- $\gamma\delta$  and TCR- $\alpha\beta$ 1 (TCR1 and TCR2 respectively). Almost all  $\alpha\beta$ 1-TCR+ cells co-expressed IL-7R $\alpha$  in the medulla, but many TCR- $\gamma\delta$ + cells did not co-express chIL-7R $\alpha$  (Fig. 6A). In the spleen, the FACS analysis results were confirmed. Many CD4+ cells but fewer CD8 $\alpha$ + cells co-expressed chIL-7R $\alpha$ , whereas most TCR- $\alpha\beta$ 1+ cells also co-expressed IL-7R $\alpha$  (Fig. 6B). Few TCR- $\gamma\delta$ + cells expressed the chIL-7R $\alpha$  (Fig. 6).

#### 4. Discussion

Herein we describe the cloning, characterisation, and expression patterns of the chicken IL-7R $\alpha$ . Like mammalian IL-7R $\alpha$ , the chIL-7R $\alpha$  protein sequence contains a GXWSXWS motif located in the FN\_III domain. This motif is conserved among type I cytokine receptor family members. Moreover, a small membrane proximal domain termed “Box 1” (Val<sup>277</sup> to His<sup>284</sup>) and an 11 aa motif containing two tyrosine residues (Tyr<sup>450</sup> and Tyr<sup>457</sup>) were also conserved. All these features strongly suggested that the cDNA described in this paper encodes the alpha chain of chIL-7R. The estimated molecular mass of chIL-7R $\alpha$  was 52.2 kDa.

The IL-7R consists of two components, the IL-7R $\alpha$  and a  $\gamma\text{c}$ . Both chains are essential for the biological effects of IL-7 [19], as homodimerisation of chimeric IL-7R $\alpha$  is not sufficient for signalling [20]. The model of IL-7R signalling is presumed to resemble that of other  $\gamma\text{c}$  family cytokines, with IL-2R $\beta$  being best studied [21,22]. IL-7 first binds to IL-7R $\alpha$  and then recruits  $\gamma\text{c}$ , bringing together their intracellular domains bearing Jak1 and Jak3. After mutual phosphorylation, these kinases acquire much higher activity and phosphorylate the critical Y449 (as numbered in the murine sequence) site on the IL-7R $\alpha$ . This site binds STAT5 and possibly other adaptors and in turn becomes phosphorylated by Jak1 and/or Jak3. Jiang et al. [23] showed that deletion of Box 1 eliminated Jak1 phosphorylation and mutation of Y449 eliminated



**Fig. 6.** Immunocytochemical double-staining of chIL-7R $\alpha$  (red) and T cell markers (blue) in cryosections of thymus (A) and spleen (B). Double-staining with anti-chIL-7R $\alpha$  and CD8a (panel 1), TCR- $\alpha\beta$ 1 (panel 2, or TCR- $\gamma\delta$  (panel 3). A. Except for the outer cell layers ( $\leftrightarrow$ ), most thymocytes in the cortex (C) and in the medulla (M) co-expressed chIL-7R $\alpha$  and CD8 $\alpha$ . TCR- $\alpha\beta$ 1+ cells co-expressed the chIL7R $\alpha$ , whereas TCR- $\gamma\delta$ + cells showed lower levels of co-expression with chIL-7R $\alpha$ . B. In the T cells areas around the arterioles (triangle) of the spleen, some CD8 $\alpha$ + cells, most TCR- $\alpha\beta$ 1+ cells and only few TCR- $\gamma\delta$ + cells co-expressed chIL-7R $\alpha$ .



STAT5 phosphorylation. Both Box 1 and Y449 played a critical role in generation of thymocytes *in vivo* [23]. The  $\text{chIL-7R}\alpha$  also contains a Box 1 domain and a tyrosine-containing motif with two tyrosines (Y450 and Y457) with conserved spacing between them when compared to the same motifs in mammalian and fish  $\text{IL-7R}\alpha$ . Though not proven, based on the sequence homology of the  $\text{IL-7R}\alpha$  it is likely that these signalling pathways will be conserved in the chicken, especially as Jak1, Jak3 and STAT5 are all present in the chicken genome.

In the chicken,  $\text{IL-7R}\alpha$  mRNA expression in primary and secondary lymphoid organs of embryos increased during development (data not shown) and at ED20 high mRNA expression levels were seen, especially in lymphoid organs rich in T cells. In splenic cell subpopulations, the highest mRNA expression levels were seen in T cell subpopulations, in  $\text{CD4}^+$ ,  $\text{CD8}\alpha^+$  and  $\text{CD8}\beta^+$  cells, which coincides with the expression pattern of the  $\text{IL-7R}\alpha$  in mammals.

Subsequently, expression of the  $\text{chIL-7R}\alpha$  protein was investigated on PBMC and splenocytes using a newly developed monoclonal antibody, 8F10E11. In blood, most  $\text{CD4}^+$  cells expressed  $\text{chIL-7R}\alpha$  independent of the age of the birds. In contrast, the percentage of  $\text{CD8}\alpha^+$  cells expressing  $\text{chIL-7R}\alpha$  in blood decreased with increasing age. On these cells  $\text{chIL-7R}\alpha$  was mostly expressed on  $\text{CD8}\alpha^{\text{int}}$  cells. Interestingly, these cells rarely co-expressed  $\text{CD8}\beta$ , indicating that although  $\text{IL-7R}\alpha$  mRNA was expressed in  $\text{CD8}\beta^+$  cells, the protein was not expressed on the surface of the cells. Stimulation of splenocytes did not induce expression of  $\text{chIL-7R}\alpha$  on  $\text{CD8}\beta^+$  cells (data not shown). The finding that mRNA transcripts are found in  $\text{CD8}\beta^+$  cells, but extracellular protein expression cannot be detected, needs further investigation, which will be focussed on intracellular expression of  $\text{IL-7R}\alpha$  and antigen-specific stimulation of  $\text{CD8}\beta^+$  cells. The expression of  $\text{chIL-7R}\alpha$  on splenic  $\text{CD4}^+$  and  $\text{CD8}\alpha^+$  cells was comparable to PBMC, except for the percentage of positive cells. A significantly lower percentage of  $\text{CD4}^+$  and  $\text{CD8}\alpha^+$  spleen cells co-expressed the receptor, with no effect of age on the  $\text{CD4}^+$   $\text{IL-7R}\alpha^+$  cells and a decreasing percentage of  $\text{CD8}\alpha^+$   $\text{IL-7R}\alpha^+$  cells with increasing age. In both peripheral blood and spleen there was no preferential expression of  $\text{chIL-7R}\alpha$  on either  $\text{TCR-}\alpha\beta 1^+$  or  $\text{TCR-}\alpha\beta 2^+$  cells. The lower percentage of  $\text{chIL-7R}\alpha^+$  T cells in the spleen might indicate that a higher percentage of T cells are activated in this organ.

Levels of  $\text{CD8}\alpha$  expression inversely correlated with expression levels of  $\text{IL-7R}\alpha$  (Fig. 4B), in that  $\text{CD8}\alpha^{\text{int}}$  cells expressed  $\text{chIL-7R}\alpha$  and most  $\text{CD8}\alpha^{\text{hi}}$  cells did not. The  $\text{CD8}\alpha^{\text{int}}$  cells mostly express the  $\text{CD8}\alpha\alpha$  homodimer and not the  $\text{CD8}\alpha\beta$  heterodimer. Especially in blood, some of the  $\text{CD8}\alpha^{\text{int}}$  cells were  $\text{CD4}^+$   $\text{CD8}\alpha\alpha^+$ . In chickens,  $\text{CD8}\alpha\alpha$  homodimers have to date been described on NK cells [24] and intestinal intraepithelial lymphocytes [25]. In mice,  $\text{CD8}\alpha\alpha$  is constitutively expressed on intraepithelial lymphocytes [26], but not uniquely on mucosal T cells.  $\text{CD8}\alpha\alpha$  is not expressed on resting naïve T cells in the periphery, but co-expression of  $\text{CD8}\alpha\alpha$  and  $\text{CD8}\alpha\beta$  on activated conventional T cells has been reported [27,28]. The induction of  $\text{CD8}\alpha\alpha$  on primary effector cells *in vivo* is not an activation marker, but  $\text{CD8}\alpha\alpha$  plays a role during initial survival and differentiation of the memory precursor. Consistent with memory precursors, these  $\text{CD8}\alpha\alpha^+$  cells co-expressed high levels of  $\text{IL-7R}$ , which is characteristic for memory T cells and their predecessors [29,30]. Using cytotoxic T cell lines, the expression of both  $\text{CD8}$  isoforms was shown to be differentially regulated. In response to TCR activation *in vitro*  $\text{CD8}\alpha\beta$  is down-regulated and internalised together with the activated TCR, whereas  $\text{CD8}\alpha\alpha$  is up-regulated [30]. Although it is unclear as to what occurs to  $\text{CD8}$  isoforms upon TCR stimulation in the chicken, the  $\text{CD8}\alpha\alpha^+$  cells in blood and spleen are relatively more intracellular  $\text{IFN-}\gamma^+$  than the  $\text{CD8}\alpha^{\text{hi}}$  population [13], which could indicate that these cells have a memory or predecessor

phenotype in the chicken too. Luhtala et al. [31] demonstrated that chicken  $\text{CD4}^+$   $\text{CD8}\alpha\alpha^+$  cells are functionally normal T cells, since they proliferate in response to mitogens and signals delivered via the  $\text{TCR-}\alpha\beta$  as well as via the  $\text{CD28}$  co-receptor.

In mammals,  $\text{IL-7R}\alpha$  transcription is suppressed in response to  $\text{IL-7}$ , to other pro-survival cytokines ( $\text{IL-2}$ ,  $\text{IL-4}$ ,  $\text{IL-6}$ , and  $\text{IL-15}$ ) and upon antigen encounter. Consequently,  $\text{IL-7R}$  expression is reduced and these T cells undergo massive clonal proliferation and differentiation into effector cells, and do not compete with unstimulated T cells for any remaining  $\text{IL-7}$ . Chicken splenocytes were polyclonally stimulated with ConA and the expression of  $\text{chIL-7R}\alpha$  decreased significantly thereafter. Considering that the number of  $\text{CD4}^+$  and  $\text{CD8}\alpha^+$  cells increased after stimulation and the geometric MFI of  $\text{CD4}^+$  and  $\text{CD8}\alpha^+$  staining slightly decreased, the down-regulation of  $\text{chIL-7R}\alpha$  cell surface expression was unlikely to be due to loss of  $\text{CD4}^+$  and  $\text{CD8}\alpha^+$  cells.

The immunocytochemical data confirmed the  $\text{chIL-7R}\alpha$  expression patterns shown by FACS analysis, indicating that we developed a useful tool to detect chicken  $\text{IL-7R}\alpha$  that can be applied in different techniques, thereby expanding the chicken's "immunological toolbox". Future research will focus on chicken T cell development and discrimination between functional T cell subsets.

## Acknowledgements

L. Vervelde and D. van Haarlem were financially supported by a BSIK VIRGO consortium grant (Grant No. 03012), The Netherlands and the Program "Impulse Veterinary Avian Influenza Research in the Netherlands", Dutch Ministry Agriculture, Nature & Food Quality. P. Kaiser and L. Rothwell were supported by core strategic grant from the Biotechnology and Biological Sciences Research Council and as part of the BBSRC/SEERAD Immunological Toolbox (grant numbers BBS/B/00255, MRI/094/04).

## References

- [1] Namen AE, Schmierer AE, March CJ, Overell RW, Park LS, Urdal DL, et al. B cell precursor growth-promoting activity: purification and characterisation of a growth factor active on lymphocyte precursors. *J Exp Med* 1988;167(3):988–1002.
- [2] Watson JD, Morissey PJ, Namen AE, Conlon PJ, Widmer MB. Effect of  $\text{IL-7}$  on the growth of fetal thymocytes in culture. *J Immunol* 1989;143(4):1215–22.
- [3] Bradley LM, Haynes L, Swain SL.  $\text{IL-7}$ : maintaining T-cell memory and achieving homeostasis. *Trends Immunol* 2005;26(3):172–6.
- [4] Murakami M, Narazaki M, Hibi M, Yawata H, Yasukawa K, Hamaguchi M, et al. Critical cytoplasmic region of the interleukin 6 signal transducer gp130 is conserved in the cytokine receptor family. *Proc Natl Acad Sci USA* 1991;88(24):11349–53.
- [5] Mazzucchelli R, Durum SK. Interleukin-7 receptor expression: intelligent design. *Nat Rev Immunol* 2007;7(2):144–54.
- [6] Huster KM, Busch V, Schiemann M, Linkemann K, Kerksiek KM, Wagner H, et al. Selective expression of  $\text{IL-7}$  receptor on memory T cells identifies early  $\text{CD40L}$ -dependent generation of distinct  $\text{CD8}^+$  memory T cell subsets. *Proc Natl Acad Sci USA* 2004;101(15):5610–5.
- [7] Van Leeuwen EM, de Bree GJ, Remmerswaal EB, Yong SL, Tesselaar K, ten Berge IJ, et al.  $\text{IL-7}$  receptor alpha chain expression distinguishes functional subsets of virus-specific human  $\text{CD8}^+$  T cells. *Blood* 2005;106(6):2091–8.
- [8] Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, et al. Expression of interleukin ( $\text{IL}$ )-2 and  $\text{IL-7}$  receptors discriminates between human regulatory and activated T cells. *J Exp Med* 2006;203(7):1693–700.
- [9] Geissen M, Heller S, Pennica D, Ernsberger U, Rohrer H. The specification of sympathetic neurotransmitter phenotype depends on gp130 cytokine receptor signaling. *Development* 1998;125(23):4791–801.
- [10] Min W, Lillehoj HS, Fetterer RH. Identification of an alternatively spliced isoform of the common cytokine receptor gamma chain in chickens. *Biochem Biophys Res Commun* 2002;299(2):321–7.
- [11] Kaiser P, Stäheli P. Avian cytokines and chemokines. In: Davison F, Kaspers B, Schat KA, editors. *Avian Immunology*. London, San Diego CA: Elsevier Ltd.; 2008. p. 203–22.
- [12] Kaiser P, Poh TY, Rothwell L, Avery S, Balu S, Pathania US, et al. A genomic analysis of chicken cytokines and chemokines. *J Interferon Cytokine Res* 2005;25(8):467–84.
- [13] Ariaans MP, van de Haar PM, Lowenthal JW, van Eden W, Hensen EJ, Vervelde L. ELISPOT and intracellular cytokine staining: novel assays for

- quantifying T cell responses in the chicken. *Dev Comp Immunol* 2008;32(11):1398–404.
- [14] Eldaghayes I, Rothwell L, Williams A, Withers D, Balu S, Davison F, et al. Infectious bursal disease virus: strains that differ in virulence differentially modulate the innate immune response to infection in the chicken bursa. *Viral Immunol* 2006;19(1):83–91.
- [15] Segers RP, De Nijs A, van Kooten PJ, Gaastra W, van der Zeijst BA. The use of immunodeficient male (CBA/N × BALB/c) F1 mice to produce monoclonal antibodies directed to proteins of *Leptospira interrogans* rather than to immunodominant lipopolysaccharides. *Hybridoma* 1990;9(3):275–83.
- [16] Vervelde L, Jeurissen SHM. Postnatal development of intra-epithelial leucocytes in the chicken digestive tract: phenotypical characterization *in situ*. *Cell Tissue Res* 1993;274(2):295–301.
- [17] Vervelde L, Vermeulen AN, Jeurissen SHM. *In situ* immunocytochemical detection of cells containing antibodies specific for *Eimeria tenella* antigens. *J Immunol Meth* 1992;151(1–2):191–9.
- [18] Bucy RP, Chen CH, Cooper MD. Ontogeny of T cell receptors in the chicken thymus. *J Immunol* 1990;144(4):1161–8.
- [19] Lai L, Goldschneider I. Cutting Edge: identification of a hybrid cytokine consisting of IL-7 and the beta-chain of the hepatocyte growth factor/scatter factor. *J Immunol* 2001;167(7):3550–4.
- [20] Ziegler SE, Morella KK, Anderson D, Kumaki N, Leonard WJ, Cosman D, et al. Reconstitution of a functional interleukin (IL)-7 receptor demonstrates that the IL-2 receptor gamma chain is required for IL-7 signal transduction. *Eur J Immunol* 1995;25(2):399–404.
- [21] Higuchi M, Asao H, Tanaka N, Oda K, Takeshita T, Nakamura M, et al. Dispensability of Jak1 tyrosine kinase for interleukin-2-induced cell growth signalling in a human T cell line. *Eur J Immunol* 1996;26(6):1322–7.
- [22] Miyazaki T, Kawahara A, Fujii H, Nakagawa Y, Minami Y, Liu ZJ, et al. Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. *Science* 1994;266(5187):1045–7.
- [23] Jiang Q, Li WQ, Hofmeister RR, Young HA, Hodge DR, Keller JR, et al. Distinct regions of the interleukin 7 receptor regulate different Bcl2 family members. *Mol Cell Biol* 2004;24(14):6501–13.
- [24] Göbel TW, Chen CL, Shrimpf J, Grossi CE, Bernot A, Bucy RP, et al. Characterization of avian natural killer cells and their intracellular CD3 protein complex. *Eur J Immunol* 1994;24(7):1685–91.
- [25] Tregaskes CA, Kong FK, Paramithiotis E, Chen CL, Ratcliffe MJ, Davison TF, et al. Identification and analysis of the expression of CD8 alpha beta and CD8 alpha alpha isoforms in chickens reveals a major TCR-gamma delta CD8 alpha beta subset of intestinal intraepithelial lymphocytes. *J Immunol* 1995;154(9):4485–94.
- [26] Rocha B, Vassalli P, Guy-Grand D. The V-beta repertoire of mouse gut homodimeric alpha CD8+ intraepithelial T cell receptor alpha/beta + lymphocytes reveals a major extrathymic pathway of T cell differentiation. *J Exp Med* 1991;173(2):483–6.
- [27] Leishman AJ, Naidenko OV, Attinger A, Koning F, Lena CJ, Xiong Y, et al. T cell responses modulated through interaction between CD8alpha alpha and the nonclassical MHC I molecule, TL. *Science* 2001;294(5548):1936–9.
- [28] Madakamutl L, Christen U, Lena CJ, Wang-Zhu Y, Attinger A, Ellmeier W, et al. CD8αα mediates survival and differentiation of CD8 memory T cell precursors. *Science* 2004;304(5670):590–3.
- [29] Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. *Nat Immunol* 2003;4(12):1191–8.
- [30] Cawthon AG, Lu H, Alexander-Miller MA. Peptide requirement for CTL activation reflects the sensitivity to CD3 engagement: correlation with CD8alphabeta versus CD8alphaalpha expression. *J Immunol* 2001;167(5):2577–84.
- [31] Luhtala M, Lassila O, Toivanen P, Vainio O. A novel peripheral CD4+ CD8+ T cell population: inheritance of CD8alpha expression on CD4+ T cells. *Eur J Immunol* 1997;27(1):189–93.